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EFFECTS OF MILK PRODUCTION AND SOME BLOOD METABOLITES ON PREGNANCY STABILITY IN LACTATING HOLSTEIN DAIRY COWS

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Abstract. In this experiment, relationships and correlations among some blood metabolites associated with energy and protein balance and their effects on pregnancy failure were investigated in lactating Holstein dairy cows. Blood and milk samples were collected from fifty-six lactating Holstein dairy cows based on their reproductive status (in 35–45 d post AI) and blood urea, milk urea, glucose, triglyceride, cholesterol and betahydroxy butyric acid were measured by enzymatic colorimetric and blood progesterone was measured by ELISA method. Cows at 56 to 63 day post Artificial Insemination, were divided in to two groups of Pregnant (PG; n=25) and Non–pregnant (NPG; n=31) by touché rectal pregnancy diagnosis. In NPG group, there were significant correlations among milk yield and some energy balance related metabolites (betahydroxy butyric acid and glucose) concentrations and correlations among milk yield and protein balance related metabolites (blood urea and milk urea) were significant in both NPG and PG groups. Furthermore, there were significant differences between means of monthly milk yield record, cholesterol, betahydroxy butyric acid and progesterone between two groups. In conclusion, although evidence exists for adverse effects of elevated circulating urea on fertility, pregnant cows were able to adapt to elevated circulating urea over several days. However fluctuating concentrations of metabolites in the early postpartum period may offer potential explanations for latent effects of early postpartum energy balance on subsequent fertility. Furthermore, in pregnant cows, the importance of progesterone and cholesterol as a precursor of ovarian steroidogenesis for pregnancy stability has been observed.
Keyword*:* : biomarker, blood metabolites, energy balance, pregnancy.

Introduction

Genetic selection programs based solely on increased milk production have resulted in cows that are genetically predisposed to a greater degree of Negative Energy Balance (NEB) in early lactation. The NEB occurs because increased Dry Matter Intake (DMI) accounts for only about half of the milk yield response to selection.

Additional substrate required to support milk synthesis is provided through enhanced mobilization of adipose reserves and skeletal muscle [VEERKAMP, 1998].

An antagonistic relationship exists between genetic merit for milk yield and reproduction, with increasing NEB in early lactation being cited as an underlying causal factor [JORRITSMA *et al.*, 2003; PRYCE *et al.*, 2004].

Failure to establish a successful pregnancy may arise from failure of cows to exhibit estrus, failure to establish an appropriate pattern of ovarian steroidogenesis and follicular growth, or from embryo mortality [ROYAL *et al.*, 2000].

Increasing severity of NEB in early lactation is associated with impaired ovarian function and delayed resumption of estrous cycles [JOLLY *et al.*, 1995].

Recently in an extensive point of view, NEB and other metabolic and hormonal imbalances proposed as

Physiological Imbalance (PI). PI defined as cows whose physiological parameters deviate from the normal and who consequently have an increased risk

of developing production diseases (clinical or subclinical) and reduced production or reproduction [INGVARTSEN, 2006].

Reducing the degree of PI in individual cows cause to reduce the risk of disease and, thereby, improve production and reproductive performance [INGVARTSEN, 2006; MOYES *et al.*, 2010].

Estimation of energy status under field conditions is difficult, because energy content of the feed depends highly on environmental factors, such as climate, processing, and storage.

Furthermore, estimates of DMI are inaccurate because there is variation with physiological state of the individual cow, ambient temperature, photoperiod, feeding strategy, and forage quality [ALLEN, 2000; INGVARTSEN and ANDERSEN, 2000].

In addition, digestibility of feed and of energy–yielding substances and the efficiency of their further use depend on many factors.

Therefore, assessments of energy ingested at the animal level might provide more reliable information.

Studies have shown that some metabolites that characterize NEB are associated with reduced fertility [WALSH *et al.*, 2011].

Several reviews [INGVARTSEN *et al.*, 2003; INGVARTSEN, 2006; LeBLANC, 2010] identified plasma NEFA, BHBA, and glucose as the major metabolites that relate to degree of PI.

However, evaluating the benefits of including other variables in blood, Blood Urea (BU) or Milk Urea (MU), cholesterol and triglyceride in the index for PI to improve the use of PI as a risk factor for disease and reproductive failure is warranted [MOYES *et al.*, 2013; BJERRE–HARPOTH *et al.*, 2012].

Previous studies have focused solely on effect of NEB or protein nutrition on reproductive performance; but in this study we investigate effect of both energy and protein related metabolites on pregnancy status of lactating Holstein dairy cows.

Therefore the objectives of this study were:

- to determine relationships and correlation among some blood metabolites that based on previous studies are related to energy and protein balance; and
- to determine their effects on pregnancy failure in high producing lactating dairy cows.

The hypotheses were:

- there is correlation between milk production and protein and energy balance related metabolites; and
- there is difference between mean of these metabolites in different groups based on their reproductive status.

Material and methods

Farm Management

A commercial dairy farm in North West of Iran, Tabriz, with 1300 lactating Holstein cows was used in this study. Cows were housed in a free stall system and fed a Total Mixed Ration (TMR).

The same diet was fed to all milking herds, except cows in their first 20 days postpartum, which were fed a ration containing additional effective fibers.

Cows were milked 3 times daily (at approximately 0530, 1330, and 2130 h) and fed following each milking.

For determining diet chemical composition, 5 random samples were collected (approximately every 3 to 4 weeks) during experimental period and were analyzed in feed analysis laboratory. Samples prepared for analysis according AOAC [AOAC 2000, method 934.01] and Dry Matter, Crude Protein according AOAC [AOAC 2000 method 976.5] by Kjeldatherm, Gerhardt; Germany, Etheric Extract according AOAC [AOAC 2000, method 920.39] by Soxtherm, Gerhardt; Germany; Crude Fiber according AOAC [AOAC 2000, method 969.09] Ash and Nitrogen Free Extract were analysed [BUTU, *et al.*, 2014, RODINO, *et al.*, 2014, BUTU, *et al.*, 2015, BUTNARIU, *et al.*, 2015].

Results were shown in Table 1.

Reproductive management consisted of a voluntary waiting period of 60 days.

After that cows were identified in estrus by visual observation.

Pregnancy diagnosis was performed by herd veterinarians by touché rectal method approximately 56 to 63 d after insemination.

Experimental Animals

Fifty-six Holstein dairy cows (16 at first, 14 at second, 9 at third and 17at fourth and higher Parity) were used in the study.

They were at 70–130 DIM. Blood and milk samples were collected during 4 months (August to December) based on cows reproductive status.

Cows that they didn't return to estrus between 18–25 days post AI, were sampled in 35–45 days post AI (approximately at 1000 h after milking and feeding).

Cows approximately at 56 to 63 days post AI, divided in to two groups of Pregnant (PG; n=25) and Non–pregnant (NPG; n=31) by pregnancy diagnosis that were determined with touché rectal method by herd veterinarian specialist.

Sampling and Measurements

Blood samples were collected from tail vein at approximately 1000h (after milking and feeding time) by evacuated Venoject tubes (without anti-coagulant), placed on Ice, carried to the laboratory and centrifuged (15 Min at 3500 rpm) and reserved in 2 cc micro tubes at –20°C.

At the same time milk samples were collected from one cartier by 50cc Falcon capped vials, placed in +4°C refrigerator carried to the laboratory and milk serum was separated by TCA (Tri Chloro acetic Acid) method and reserved at –20°C.

Blood serum glucose, urea, triglyceride, cholesterol and milk serum urea were measured by enzymatic colorimetric method with spectrophotometer (Shimadzo; Japan) and Ziest Chem kits. Blood serum Beta Hydroxy Butiric Acid (BHBA) was measured by Ultra Violet (UV) method with spectrophotometer and Ranbut kits (RANDOX laboratories; UK, RT294QY).

Blood serum progesterone was measured by ELISA method with ELISA Reader (Anthos 2020; Salzburg, Austria) and DRG ELISA kits (EIA–1561 DRG Instruments GmbH, Germany, 18, D–35039 Marburg).

Statistical Analysis

One of the objectives of this study was to determine the relationships between variables that based on previous studies are related to energy and protein balance.

For this propose before correlation analysis, measured data tested for skewness and kurtosis. In spite of significant test results, there was no difference in correlation analysis of converted and measured data.

Therefore, measured data were used in the statistical analysis.

Another objective of this study was to determine effect of variables that related to energy and protein balance on reproduction and pregnancy status that was performed by variables means comparison by t–Test in two groups that were divided by cow's reproductive statues. SAS 8.1 and SPSS 19 soft wears were used for statistical analysis.

Results and discussion

As mentioned previously, blood and milk samples were collected from cows in 35–45 days post AI and divided into two groups of pregnant (PG; n=25) and Non–pregnant (NPG; n=31) by pregnancy diagnosis that were determined with touché rectal method by herd veterinarian specialist.

Correlation Coefficients

Simple correlation coefficients among variables in NPG group were shown in Table 2.

In this group, there was negative correlation between Days in Milk (DIM) and Monthly Milk Yield (MMY) ($r=–0.5893$; $p<0.001$), Blood Urea concentration (BU) ($r=–0.5187$; $p<0.01$), Milk Urea concentration (MU) ($r=–0.4310$; $p<0.05$), Beta Hydroxy Butyric Acid (BHBA) ($r=–0.3891$; $p<0.05$) and positive correlation between DIM and blood Glucose concentration ($r=0.4570$; $p<0.01$).

Also, positive correlation between MMY and BU ($r=0.5692$; $p<0.001$), MU ($r=0.4951$; $p<0.001$), BHBA ($r=0.5089$; $p<0.01$) and negative correlation between MMY and Glucose ($r=–0.6668$; $p<0.001$) were observed.

Furthermore BU was positively correlated with MU($r=0.9030$; $p<0.001$) and with BHBA ($r=0.5475$; $p<0.01$) but negatively correlated with Glucose (r=–

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0.7565; p<0.001) and with Cholesterol (r=–0.3792; p<0.05).

Correlation of MU with glucose also was negative (r=–0.7063; p<0.001) and like BU, there was positive correlation between MU and BHBA (r=0.4107; p<0.05). In this group Glucose was negatively correlated with BHBA (r=–0.4908; p<0.01).

Table 1.

Ingredients and chemical composition of diet fed during study on dairy cows

Ingredient	
Alfalfa hay; chopped, g/kg of DM	218
Corn silage, g/kg of DM	209
Barley, g/kg of DM	221.2
Cottonseed meal, g/kg of DM	120.4
Wheat bran, g/kg of DM	100
Corn; whole grain g/kg of DM	48
Cottonseed; whole grain, g/kg of DM	30
Corn gluten meal, g/kg of DM	24
Sugar beet pulp, g/kg of DM	12
Dicalcium phosphate, g/kg of DM	8.7
Vitamin and Mineral mix, g/kg of DM	5.8
Salt, g/kg of DM	2.9

Chemical composition	Mean	SD	Max	Min
Dry matter %	55.81	1.17	57.33	54.11
Crude protein %	18.25	0.84	18.83	16.68
Crude fiber %	41.59	0.74	42.65	40.57
Ether extract %	5.97	0.11	6.13	5.83
Ash %	10.13	0.48	10.64	9.37
Nitrogen free extract %	23.96	0.21	24.29	23.73

*Net Energy for Lactation, 1.55 Mcal/kg of DM.
*Vitamin and Mineral mix contained a minimum of 90000 mg/kg of P, 180000 mg/kg of Ca, 60000 mg/kg of Na, 25000 mg/kg of Mn, 3000 mg/kg of Cu, 2000 mg/kg of Mg, 3000 mg/kg of Zn, 3000 mg/kg of Fe, 100 mg/kg of Co, 30 mg/kg of Se, 200 mg/kg of I, 3000 mg/kg of Antioxidant, 560000 IU/kg vitamin A, 100600 IU/kg vitamin D3 and 1030 IU/kg vitamin E.

Simple correlations among variables in PG group were shown in Table 3.

In this group, there was negative correlation between DIM and MMY (r=–0.7926; p<0.001) and BU (r=–0.5117; p<0.01) and positive correlation between MMY and BU (r=0.7303; p<0.001) and MU (r=0.5519; p<0.01).

Also BU was positively correlated with MU (r=0.7011; p<0.001).

Furthermore, there was positive correlation between Triglyceride (r=0.3995; p<0.05).

Comparison of Means (t–Test)

Means were compared by t–Test exam, which results are shown in Table 4. There was deference between MMY (NPG=30.47, PG= 26.22 kg; t=–5.560, p<0.001), Cholesterol (NPG=2.6748, PG=2.9768 mmol/L; t=2.093, p<0.05), BHBA (NPG=0.3948, PG=0.3556 mmol/L; t=–3.765, p<0.001) and Progesterone (NPG=4.4777, PG=16.1084 ng/dL; t=9.254, p<0.001).

Major changes in the endocrine, immune, and digestive systems, for example, occur around parturition and play vital roles in the transition from pregnancy to lactation [INGVARTSEN, 2006].

Deviations from normal for any of these physiological systems increases degree of PI and thereby risk of disease, especially during early lactation [INGVARTSEN and MOYES, 2013].

Links exist between metabolic maintenance and reproductive efficiencies that involve the complex integration of endocrine and metabolic signals controlling metabolism and reproduction.

Whatever the mechanism, it is clear that the reproductive success of the dairy cow is linked to body energy reserves and metabolic responses to nutrition [ROCHE, 2006].

These responses must inevitably involve signaling molecules and hormones that are integral components of the control systems that regulate the partitioning of energy and nutrients and the reproductive axis.

When they are identified, these signaling molecules will be produced by the many sites (organs) that are involved in processing, storage, and utilization of nutrients, or in the reproductive process.

Moreover, to ensure precise regulation of energy and protein partitioning, and integration with the reproductive process, any given signal probably interacts with other signals [CHANGAS *et al.*, 2007].

However biomarkers that accurately reflect changes in the endocrine, immune, and digestive systems, and thereby degree of PI, during lactation are lacking [MOYES *et al.*, 2013].

In this study relationships and correlation among some blood metabolites that based on previous studies are related to PI–BU and MU as an adequate indicator of metabolic status, especially in the early lactation [WATTIAUX and KARG, 2004] and glucose, triglyceride, cholesterol and BHBA as indicators of energy balance [MOYES *et al.*, 2013; BJERRE–HARPOTH *et al.*, 2012]–were investigated with considering some animal variables DIM, MMY and parity within cows that they failed to continue reproductive persistency, Non–Pregnant Group (NPG) and within cows that they continue a stable reproductive status, Pregnant Group (PG).

Table 2.

Simple Correlations Coefficients among Animal Variables (DIM, MMY, and Parity), Protein Balance Related Metabolites (BU and MU) and Energy Balance Related Metabolites (Glucose, TG, Cholesterol and BHBA) in NPG group (n=31).

Variable	DIM	Parity	MMY	BU	MU	Glucose	TG	Cholesterol	BHBA
DIM	1								
Parity	0.0323	1							
P–Value	0.8631								
MMY	**–0.5893*****	–0.0418	1						
P–Value	0.0005	0.8231							
BU	**–0.5187****	0.1901	**0.5692*****	1					
P–Value	0.0028	0.3056	0.0008						
MU	**–0.4310***	0.2742	**0.4951*****	**0.9030*****	1				
P–Value	0.0155	0.1355	0.0046	<0.0001					
Glucose	**0.4570****	–0.1780	**–0.6668*****	**–0.7565*****	**–0.70631*****	1			
P–Value	0.0098	0.3379	<0.0001	<0.0001	<0.0001				
TG	–0.0757	0.1204	0.2972	0.0361	0.0523	–0.1157	1		
P–Value	0.6855	0.5187	0.1044	0.8470	0.7796	0.5354			
Cholesterol	0.1030	–0.0892	–0.0914	**–0.3792***	**–**0.3231	0.3146	0.0182	1	
P–Value	0.5811	0.6332	0.6246	0.0354	0.0762	0.0847	0.9224		
BHBA	**–0.3890***	0.0646	**0.5089****	**0.5475****	**0.4107***	**–0.4908****	–0.1273	0.0375	1
P–Value	0.0305	0.7298	0.0035	0.0014	0.0217	0.0051	0.4949	0.8412	

DIM= Days in Milk; MMY= Monthly Milk Yield; BU = Blood Urea; MU=Milk Urea; TG= Triglycerides; BHBA= Betahydroxy Butyric Acid, Values in bold are significant. **P< 0.05; **P<0.01; ***P<0.001*.

As shown in the Tables 2 and 3 there were some expected similar results for both NPG and PG groups.

There was a positive correlation between MU and BU for both NPG (r= 0.9031; p<0.001) and PG (r=0.7011; p<0.001) groups, that is similar to the results of Broderick and Clayton and Hof and collab. [BRODERICK and CLAYTON, 1997; HOF *et al.* 1997].

These results were expected because urea is the metabolic end product of protein catabolism in the body and is easily measured by the nitrogen content (i.e., urea nitrogen concentration).

Urea nitrogen concentrations circulating in the bloodstream are measured in either plasma or serum fractions (PU or SU, respectively) or are often referred to generically as BU.

Typically, BU peaks about 4 to 6 h after meals because of Rumen Degradable Protein (RDP) catabolism, and the metabolism of Rumen

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Undegradable Protein (RUP) contributes to BU continuously throughout the day.

The fluctuations in BU during the day are usually smaller (2 to 3 mg/100 dL) in cows fed a TMR than in cows fed concentrates and forages separately.

Urea is a small, water–soluble molecule that permeates all cells and tissues in the body and passes easily between the blood and milk within the mammary gland. With a lag time of less than 1 h for equilibration with blood concentrations MU provides a rapid, non-invasive, and inexpensive means of assessing the dynamics of BU [BUTLER, 1998].

Therefore, in the both groups high correlation of BU and MU was expected.

Table 3.

Simple Correlations Coefficients among Animal Variables (DIM, MMY, and Parity), Protein Balance Related Metabolites (BU and MU) and Energy Balance Related Metabolites (Glucose, TG, Cholesterol and BHBA) in PG group (n=25).

Variable	DIM	Parity	MMY	BU	MU	Glucose	TG	Cholesterol	BHBA
DIM	1								
Parity	–0.0505	1							
P–Value	0.8104								
MMY	**–0.7926*** **	–0.0392	1						
P–Value	<0.0001	0.8524							
BU	**–0.5117** **	–0.0594	**0.7303*** **	1					
P–Value	0.0089	0.7778	<0.0001						
MU	–0.3443	0.0311	**0.5519** **	**0.7011*** **	1				
P–Value	0.0919	0.8823	0.0042	<0.0001					
Glucose	–0.1162	–0.1106	–0.0794	–0.0939	–0.0573	1			
P–Value	0.5801	0.5984	0.7059	0.6550	0.7852				
TG	0.1169	–0.1388	–0.1496	–0.0319	0.0544	–0.0445	1		
P–Value	0.5778	0.5079	0.4754	0.8795	0.7961	0.8327			
Cholesterol	–0.0393	0.3919	0.0764	0.0160	0.0406	0.1642	**0.3995***	1	
P–Value	0.8519	0.0526	0.7163	0.9395	0.8469	0.4327	0.0478		
BHBA	–0.0348	0.0431	0.2281	0.2476	0.0801	–0.3626	–0.1940	0.0651	1
P–Value	0.8686	0.8379	0.2728	0.2326	0.7037	0.0749	0.3527	0.7571	

DIM= Days in Milk; MMY= Monthly Milk Yield; BU = Blood Urea; MU=Milk Urea; TG= Triglycerides; BHBA= Betahydroxy Butyric Acid; Values in bold are significant. **P< 0.05; **P<0.01; ***P<0.001*.

Furthermore, another expected similar result, was negative correlation between DIM and MMY in NPG (r=–0.5893; p<0.001) and PG (r=–0.7926; p<0.001) groups. It is well considered that, 305–d lactation curve has a peak in the first 30 to 60 DIM and a constant decrease over the rest of the lactation. In this study, experimental animals were at least in more than 70 DIM, therefore it is expected that by increasing DIM, MMY decrease.

Protein Balance Related Metabolites Correlation Coefficients

In this study, BU was negatively correlated with DIM in both NPG (r=–0.5187; p<0.01) and PG (r=–0.5117; p<0.01) groups and BU correlation with MMY in both NPG (r=0.5692; p<0.001) and PG (r=0.7303; p<0.001) groups was positive. Partially similar results were observed between MU and milk yield.

Reports on the association between MU and milk yield vary between positive [CARLSSON *et al.*, 1995], no association [BAKER *et al.*, 1995], and negative [ISMAIL *et al.*, 1996].

Many factors contribute to the actual blood urea concentration measured in both late–pregnant and early–lactating ruminants.

These include the degree of catabolism of AA stored in skeletal muscle to meet the requirements of the conceptus (prepartum), and mammary gland (postpartum) and dietary factors [MOORE and VARGA, 1996].

Concentration of blood urea is influenced by both the ratio and concentration of RDP and RUP and the ratio of energy to protein in the diet.

Circulating ammonia concentrations increase after degradation of RDP, particularly during energy deficit, and urea production by the liver also requires energy and may exacerbate the NEB.

Impaired liver function, as commonly occurs after calving, also reduces the metabolic clearance of urea [O'CALLAGHAN *et al.*, 2001].

Elevated blood urea also can be caused by a low rumen pH, which inhibits the growth of microorganisms, whereas reduced voluntary DMI around calving, liver failure, or both may contribute to reduced urea [MOORE and VARGA, 1996].

Therefore several recent results suggest MU as an adequate indicator of metabolic imbalance, especially in the early lactation of dairy cows.

For example, Wattiaux and Karg found that MU concentration was negatively correlated with DMI at 3 wk of lactation, and this correlation was larger in cows with a greater milk yield relative to DMI [WATTIAUX and KARG 2004].

Wattiaux and Karg concluded that MU may be more reflective of energy and protein balance in the early lactation of dairy cows than the adequacy of dietary inputs would be [WATTIAUX and KARG 2004].

However, remarkable point about urea concentration correlation with milk yield in this study is that, it was observed in both NPG and PG groups that means pregnant cows were able to adapt to elevated urea concentration by high milk yield. In a review, Laven and collab. concluded that, although evidence exists for adverse effects on fertility of elevated circulating urea, cows were able to adapt to a high dietary nitrogen input over several days [LAVEN *et al.*, 1999]. An elevated urea concentration was thus mainly indicative of an imbalance between protein and energy supply, representing another measure of NEB. At the other end of the concentration range, previous studies reported that feeding diets having inadequate protein during late gestation was associated with weak calves, stillbirths, and more retained fetal membranes [MOORE and VARGA, 1996].

All of these problems subsequently have an impact on fertility.

Energy Balance Related Metabolites Correlation Coefficients

As shown in Table 2, there was significant correlation between Glucose and DIM (r=0.4571; p<0.01), MMY (r=–0.6668; p<0.001), BU (r=–0.7565; p<0.001), MU (r=–0.7063; p<0.001) and BHBA (r=–0.4908; p<0.01) in NPG group. Furthermore in this group correlation between BHBA and DIM (r=–0.3891; p<0.05), MMY (r=0.5089; p<0.01), BU (r=0.5475; p<0.01) and MU (r=0.4107; p<0.05) was significant. But there was not any significant correlation among these variables in PG group (Table 3).

It means that in NPG group, from energy balance related metabolites, Glucose and BHBA were correlated with production variables. In fact high milk yield in these animals had an adverse effect on energy balance and as an indirect result, on reproduction. Various blood metabolites such as blood serum concentrations of glucose, BHBA, and NEFA have been reported to be strongly correlated to energy balance [REIST *et al.*, 2002; CLARK *et al.*, 2005]. Reist and collab. reported that glucose concentrations have been found to be positively correlated with energy balance [REIST *et al.*, 2002].

There is also evidence that glucose is the main energy source for the ovary [RABIEE *et al.*, 1999], whereas phenotypic studies have shown positive correlation between glucose concentrations and fertility [WESTWOOD *et al.*, 2002].

In this study in NPG group, which hypothetically was adversely affected by NEB, Glucose concentration was correlated with production variables (DIM and MMY) and with urea concentration. In some recent studies, amount and rate of glucose flow in blood were investigated during different stages of lactation.

Glucose concentrations increased slightly during the prepartum period, increased dramatically at calving, and then decreased immediately postpartum, consistent with the published literature [BUTLER *et al.*, 2006; PATTON *et al.*, 2008].

The extent of the postpartum decrease in glucose was less in lactating animals, resulting in divergent glucose concentrations between lactating and

nonlactating cows from d 3 to approximately d 49 postpartum, presumably reflective of the dramatic increase in mammary glucose requirements associated with the onset of lactation.

Voigt and collab. conducted that glucose oxidation was inversely related to milk production, indicating that less glucose is used as substrate for energy production and more glucose is available in the mammary gland for lactose synthesis in cows with high capability for milk production [VOIGT *et al.*, 2005].

A shift from glucose to fat oxidation and therefore higher glucose availability for lactose synthesis was also seen in dairy cows after rumen–protected fat feeding, pointing at the glucose sparing effect in cows with increased milk production and increased lactose synthesis.

Table 4.

Comparison Between NPG and PG measured Variables Means by t–Test method in 35 to 45 days after AI.

Variable	Unit	NPG mean	PG mean	t–Value	Significance
DIM	day	107.74	102.40	–1.126	0.265
Parity		3.48	3.04	–0.644	0.522
MMY	kg	30.47	26.22	**–5.560*** **	<0.0001
BU	mmol/L	7.2974	7.1848	–0.282	0.779
MU	mmol/L	6.8268	6.4788	–0.846	0.401
Glucose	mmol/L	2.8881	3.1668	1.324	0.191
TG	mmol/L	0.1061	0.1248	0.937	0.353
Cholesterol	mmol/L	2.6748	2.9768	**2.093***	0.041
BHBA	mmol/L	0.3948	0.3556	**–3.765*** **	<0.0001
Progesterone	ng/dL	4.4777	16.1084	**9.254*** **	<0.0001

Therefore, in general, the greater glucose demand after parturition for milk production led to decreased blood glucose concentrations after calving in all cows. Elevated blood BHBA levels are commonly used to confirm nutritional inadequacy in farm animals, and because BHBA is itself a product of NEFA metabolism by ruminant liver [ZAMMIT, 1999], unusually high BHBA values (>1.5 mmol/L) are taken to indicate low energy status and associated fat mobilization in cattle and sheep. But BHBA is also a product of butyrate metabolism by rumen epithelium and liver, which convert 75 and 60%, respectively, of butyrate passing through them to BHBA [PENNINGTON, 1952].

Rumen butyrate is increased in silage–fed ruminants offered barley–based supplements [WYLIE *et al.*, 1984], so high blood BHBA levels may not exclusively reflect mobilization of adipose reserves.

BHBA is also a substrate for de novo fatty acid synthesis in the lactating bovine mammary gland [VERNON, 2005] so that elevated BHBA levels can make a positive contribution to lactation.

In general, undernutrition is associated with a decrease in EB, suggesting an inverse correlation between EB and BHBA concentrations.

Although Reist and collab. stated that among the blood metabolites [REIST *et al.* 2002], NEFA concentration is assumed to be the best indicator of a cow's energy balance because elevated NEFA concentration is the first indication of lipolysis; in Oikonomou and collab. study it was BHBA concentrations that were found to have the strongest genetic correlation with reproductive traits [OIKONOMOU et al. 2008]. In the liver, NEFA can be esterified to triglycerides, enter the citric acid cycle, or form ketone bodies [HOLTENIUS and HOLTENIUS, 1996].

High rates of ketogenesis and high blood BHBA concentrations can be considered as cow's failure to adapt to negative energy balance [HERDT, 2000].

It is possible that cows genetically prone to lower reproductive ability are those cows that are also prone to not only mobilize greater amounts of their energy reserves but also turn a greater proportion of NEFA released from this mobilization to ketone bodies (i.e., BHBA). In other words, there might be a genetic background in the ability of dairy cows to

regulate NEFA concentrations and hence to adapt to negative energy balance; this genetic variation could explain part of the genetic relationship between energy balance and fertility.

Negative effects of high postpartum BHBA or NEFA blood concentrations on reproduction were also reported by WALSH et al. (2007), and WATHES et al. (2007). In our study, the correlation coefficient of Glucose and BHBA (r=–0.4908; p<0.01) was significant.

This suggests that Glucose had a negative relationship with the BHBA concentration. It has been reported that feeding cows glucogenic nutrients in early lactation decreases plasma BHBA and acetate concentrations [PICKETT *et al.*, 2003].

Fluctuating concentrations of metabolites in the early postpartum period may offer potential explanations for latent effects of early postpartum energy balance on subsequent fertility.

Associations between these metabolites and early lactation energy balance are well established and reflect enhanced mobilization of body reserves and partitioning of nutrients toward milk production [REIST *et al.*, 2002, FERENCZ, *et al.*, 2012].

Furthermore, the environment in which the oocyte develops can have an effect on its subsequent developmental competence. Britt hypothesized that follicles grown during the period of NEB could be affected by unfavorable metabolic changes, resulting in a latent effect on the quality of the oocyte offered for fertilization some 60 to 80 d later; in other words, conditions to which the oocyte is exposed early in lactation may be as or more important than those immediately preceding insemination.

This is based on the fact that it takes several months (60–80 d) for the oocyte to grow from the early preantral stage to the mature preovulatory follicle [BRITT, 1992; FAIR, 2010].

Comparison of Means (t–Test)

As shown in Table 4, in t–Test results for PG and NPG there was significant differences between means of MMY (t=–5.560; p<0.001), Cholesterol (t=2.093; p<0.05), BHBA (t=–3.765; p<0.001) and Progesterone (t=9.254; p<0.001). Energy balance had been shown in several studies to modulate plasma progesterone concentrations during early postpartum [SPICER *et al.*, 1993b] and concentrations of progesterone have been associated positively with fertility and pregnancy rates [SKLAN *et al.*, 1991] and negatively with EB [SPICER et al., 1993b] and days open [SKLAN *et al.*, 1991].

In addition, a negative EB reduces the weight of corpus luteum [APGAR *et al.*, 1975] and decreases steroidogenic activity of luteal tissue [VILLA–GODOY *et al.*, 1990].

Our results agree with above mentioned results because there was difference between BHBA concentration means and between progesterone concentration means of two groups and as discussed previously BHBA is one of the most reliant biomarker of NEB in postpartum period. Thus, the weekly energy status of the cows measured as the difference between net energy intake and energy expended for lactation plus maintenance in the study of Francisco and collab. served as a good predictor of return to normal ovarian activity as measured by progesterone concentrations [FRANCISCO *et al.*, 2003].

Furthermore, previously, plasma cholesterol concentrations were consistently important in predicting nutritional status of lactating and nonlactating dairy cows [KRONFELD *et al.*, 1982].

Nebel and McGillard in a study about interactions of high milk yield and reproduction performance conducted that, variables that are significantly correlated with each other include milk yield with days to first postpartum ovulation, plasma cholesterol with plasma progesterone and conception rate [NEBEL and McGILLARD, 1993].

Similarly in our study, milk yield (MMY), Cholesterol and Progesterone concentrations means were different between two groups. Previous studies have documented the importance of cholesterol as a precursor of ovarian steroidogenesis [SPICER *et al.*, 1993a]. Plasma cholesterol concentrations increase between calving and wk 6 postpartum in dairy cows, and are correlated with plasma progesterone [SPICER *et al.*, 1993b; FRANCISCO *et al.*, 2002], conception rates and

number of recoverable embryos [GRUMMER and CARROLL, 1988]. The association of increased plasma cholesterol concentration with increased luteal–phase progesterone secretion in early lactating dairy cows [SPICER et al., 1993b] merits further investigation.

Understanding what production and hormonal factors contribute to variation in plasma cholesterol may lead to insights that may help improve reproductive efficiency in dairy cattle.

Conclusions

In conclusion the result of this study suggest that in animals that they fail to continue pregnancy, or non–pregnant cows, there are significant correlations among milk yield and some energy balance related metabolites (BHBA and glucose) concentrations; however correlations among milk yield and protein balance related metabolites (BU and MU) are significant in both pregnant and non–pregnant cows, therefore we can conclude that although evidence exists for adverse effects on fertility of elevated circulating urea, pregnant cows were able to adapt to elevated circulating urea over several days.

Furthermore, in pregnant cows, the importance of progesterone and cholesterol as a precursor of ovarian steroidogenesis for pregnancy has been observed.

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